AMENDMENTS TO THE SPECIFICATION

Please amend paragraph **[0005]**, as follows:

[0005] This application is a continuation of PCT/US02/21066 with an international filing date of July 3, 2002, now abandoned, which claims the benefit of priority to U.S. Provisional Application No. 60/302,648, entitled "Coactivators in the Diagnosis and Treatment of Breast Cancer," ~~and~~ filed July 5, 2001~~, and International Patent Application No. PCT/US02/21066 of the same title and filed July 2, 2002~~. The entireties of both applications are hereby incorporated by reference.

Please amend paragraph **[0016]**, as follows:

[0016] Another embodiment of the invention is directed to isolated nucleic sequences that encode isoforms of the invention. Preferably the nucleic acid encodes the isoform [[?]] Δ3-AIB1. The invention further encompasses vectors that contains the nucleic acid of isoforms of the invention, and also recombinant cells, which may be either eukaryotic or prokaryotic, containing nucleic acids or vectors of the invention.

Please amend paragraph **[0020]**, as follows:

[0020] Another embodiment of the invention is directed to small interfering RNA molecules (siRNA) that inhibit expression of a transcriptional coactivator protein such as, for example, AIB1, isoforms of AIB1 such as the p160 group of coactivators, Src-1, Src-2, Src-3, and other isoforms, fragments and combinations thereof. Preferably, the siRNA is specifically targeted to inhibiting the expression of one protein isoform and does not target other isoforms. One preferred siRNA targets the [[?]] Δ3-AIB1 mRNA and contains sequences homologous and complementary to that mRNA. Preferably the siRNA contains a sequence, a portion of which is derived from exon 2 of the mRNA that encodes [[?]] Δ3-AIB1, and another portion of which is derived from exon 4 of the mRNA that encodes [[?]] Δ3-AIB1. The portions are each preferably from about 5 to about 16 nucleotides in length, making the total length of the RNA molecule about 12 to about 32 nucleotides long. That

sequence together with sequence complementary thereto, forms the double-stranded siRNA molecule.

Please amend paragraph **[0022]**, as follows:

[0022] Another embodiment of the invention is directed to methods for treating or preventing a tumor comprising administering to a patient a therapeutically effective dose of a pharmaceutical composition of the invention. Administration is preferably by direct injection to the tumor. These method may further [[.]] comprise administering additional tumorigenic therapy to the patient such as, for example, drug therapy, radiation therapy, surgery, and combinations thereof.

Please amend paragraph **[0023]**, as follows:

[0023] Another embodiment of the invention is directed to transgenic animals and methods for the creation of transgenic animals containing nucleic acid sequences that express AIB1 and/or isoforms, or siRNA directed against AIB1 or isoforms of the invention. Preferably the animal is a mouse and the isoform is [[?]] Δ3-AIB1.

Please amend paragraph [0025], as follows:

[0025] **Description of the Figures**

Please amend paragraph **[0034]**, as follows:

[0034] **Figure 9** **a** RT-PCR for AIB1 and [[?]] Δ3-AIB1 RNA from normal and breast cancer tissue wherein the signal between breast and normal tissue was compared using an arbitrary scale; and **b** sequence of exon junctions for AIB1 and [[?]] Δ3-AIB1 isoform **(SEQ ID NOS 1-3, respectively in order of appearance)**.

Please amend paragraph **[0038]**, as follows:

[0038] It was surprisingly discovered that an isomer of AIB1, a splice variant of AIB1 that has exon 3 deleted, the Δ3-AIB1 mRNA, is translated *in vivo* in breast cancer cells into an NH_2-terminal truncated form of AIB1. This N-terminally truncated version of AIB1 has lost most of the predicted dimerization domains and thus is more promiscuous with respect to potential interaction partners (48). The predicted size of this truncated protein is approximately 130 kDA and a protein with this molecular weight was identified in Western blot analysis of MCF-7 cells (48). In support of the significance of the N-terminal region, it was found that over expression of the [[?]] Δ3-AIB1 isoform potentiates nuclear hormone (ER, PR) and growth factor (EGF) induction of transcription to a much greater extent than full-length AIB1 (48). This indicates that over expression of this isoform in cancer plays a role in sensitizing breast cancer cells to hormone and/or growth factor induced changes in phenotype during the malignant progression of breast cancer.

Please amend paragraph **[0039]**, as follows:

[0039] [[?]] Δ3-AIB1 has several unusual properties of interest. First, on a per molecule basis, [[?]] Δ3-AIB1 is a potent transcriptional coactivator of steroid receptors such as, for example, the estrogen receptor and the progesterone receptor (see e.g. Example 4), and also growth factor signaling (e.g. see Example 5). It is also a more potent coactivator than full-length AIB1 protein as measured by conventional transcriptional assays such as, for example, a transient transfection assay (see e.g. Example 4). For a given amount of vector transfected, the amount of [[?]] Δ3-AIB1 protein produced in transient assays is only about 1% to 10% that of the full-length protein (48). Despite this, the coactivating effect of [[?]] Δ3-AIB1 is several-fold greater than full-length AIB1 making it highly effective on a molar basis. This result is unexpected given that previous studies of NH_2-terminal deletion mutants of the AIB1-related protein Src-1 did not reveal an impact of this region on nuclear receptor signaling (9, 30). One reason for the increased activity of Δ3-AIB1 is that the conformation of this isoform is more favorable than that of the full-length protein for interaction with nuclear receptors or for recruitment of other coactivators such as CBP/p300. Another reason is indicated by the observation that the bHLH-PAS domains of Src-1 interacts with and potentiates the activity of members of the TEF family of transcription factors (30). Thus, full-length AIB1 is unavailable for interaction with nuclear receptors because it is sequestered or

squelched by other intracellular factors. In contrast, AIB1-Δ3, which lacks an intact bHLH-PAS domain, would not bind to potential repressors such as TEF and would be available for nuclear receptor coactivation. This may explain why relatively small amounts of recombinant Δ3-AIB1 are able to induce significant potentiation of nuclear receptor activity in transfected COS-1 cells with a high background of endogenous full-length AIB1. This model also predicts that the relative coactivating effects of AIB1 and Δ3-AIB1 are likely cell-type specific, depending on the endogenous expression of AIB1-sequestering molecules such as TEF. A recent report described that the human MMS19 protein can interact with the PAS-HLH domain of AIB1 and can regulate ER-mediated transcriptional activity (43). The lack of interaction of Δ3-AIB1 with this protein explains some of its increased effectiveness *in vivo*. The data indicates that expression of the AIB1 isoform sensitizes cells to the effects of estrogen and progesterone.

Please amend paragraph **[0042]**, as follows:

[0042] Using ribozyme targeting that down regulates AIB1, it was determined that down regulation leads to loss of estrogen reduction and proliferation of breast cancer cells in vivo. To analyze over expression of [[?]] Δ3-AIB1 in transgenic animals, a transgenic animal was made that expressed the [[?]] Δ3-AIB1 protein under CMV control. The phenotype observed in these animals is that at about 7 weeks old, the male mice develop large mammary glands. Upon whole mount examination of these mice, it was determined that the mammary glands contain massive stromal proliferation leading to large fat tissue in the breast. Thus, the effect of the [[?]] Δ3-AIB1 isoform is to increase proliferation of stromal cells and/or increase the differentiation of fibroblasts to adipocytes. This same phenotype is seen in the female, although not as pronounced, but the stromal tissue is already quite large at seven weeks and also in other fat tissue and the stroma of the female. This indicates that the [[?]] Δ3-AIB1 isoform can play a role in aberrant expression or proliferation of stroma in breast cancer and other endocrinological malignancies and pathologies. Since a previously made AIB1 knockout mouse has been shown to be a small animal with defects in IGF signaling, the defect from the over expression of [[?]] Δ3-AIB1 is likely from over expression of IGF1 in the liver as can be determined by measuring IGF1 in the serum. These animals will likely

develop a fat phenotype later in life indicating that [[?]] Δ3-AIB1 and AIB1 have a wider role in fat metabolism. Its also possible that the effect of [[?]] Δ3-AIB1 is not mediated through IGF1, but through the PPAR system (perxoidone proliferator receptor), which is known to be central to fat metabolism.

Please amend paragraph **[0044]**, as follows:

[0044] Another embodiment of the invention is directed to isolated nucleic acid sequences that encode one or more isoforms of the invention. Preferably the nucleic acid, which may be DNA, RNA or PNA, encodes the isoform [[?]] Δ3-AIB1 containing a deletion of exon 3 from about positions 267 to 439. Please note, currently there is no accepted exon numbering. The numbering for the exons herein is based on the experiments set forth in the examples section and cited publications. However, based on the information provided herein such as specific sequence information of the AIB1 gene (Fig. 9), the locations of the binding sites along AIB1 (Fig. 1 and Fig. 9), and the transcription and translation start sites (Fig. 1 and Fig. 9), any different exon numbering system can be readily and easily correlated to those disclosed herein by those of ordinary skill in the art. The invention further encompasses vectors (e.g. plasmids, cosmids, viral, or shuttle vectors) that contains the nucleic acid of isoforms of the invention, and also recombinant cells, which may be either eukaryotic or prokaryotic (e.g. Enterobacter, *Escherichia coli*, or *Bacillus subtilis*), containing nucleic acids or vectors of the invention.

Please amend paragraph **[0048]**, as follows:

[0048] Another embodiment of the invention is directed to small interfering RNA (siRNA) molecules directed against mRNA that encode transcriptional coactivator proteins. These siRNA molecules are double-stranded and typically between about 10 bp and 50 bp in length, preferably between about 15 bp and 30 bp, and more preferably between about 18 bp and 25 bp. Methods for manufacturing such RNA molecules with any desired sequence have been previously described (see U.S. Patent No. 5,795,715), as has their use in controlling gene expression (see 51 and 55-65). These molecules are directed against transcriptional coactivators and, preferably, the transcriptional

coactivator proteins AIB1, Src-1, Src-2, Src-3, and related isoforms such as, for example, [[?]] Δ3-AIB1. The siRNA molecule directed against the [[?]] Δ3-AIB1 mRNA contains sequences that are homologous and complementary to a mRNA that encodes [[?]] Δ3-AIB1, and that hybridize to each other to form the double-stranded RNA molecule. Preferably, siRNA molecules contains sequences derived from each side of the region that is deleted such that only the particular isoform mRNA is targeted. For example, a preferred siRNA contain a sequence from a portion of exon 2 of the mRNA that encodes [[?]] Δ3-AIB1, and another portion of which is derived from exon 4 of the mRNA that encodes [[?]] Δ3-AIB1. Another preferred anti-AIB1 siRNA for identifying isoforms containing a deletion of the PAS A binding site contains a sequence wherein a portion is derived from the sequence on one side of the PAS A binding site and another portion is derived from a sequence on the other side of the PAS A binding site. Preferably the portions are similar in length to provide good interaction across the deletion and contiguous in the resulting mRNA, but may be non-contiguous containing 1, 2, 3, 4, 5, 6, or more nucleotides between the portions. Alternatively, siRNAs may contain sequences that represent additions or new sequences of the isoform mRNA not found in the wild-type mRNA. Portions, and typically the corresponding sequences of the siRNA, are each preferably from about 4 to about 20 nucleotides in length, more preferably from about 6 to 16 nucleotides in length, and more preferably from about 8 to 12 nucleotides in length. Most preferably, the siRNA is capable of targeting one or a small number of isoforms of the particular coactivator, but not the wild-type coactivator mRNA. The greater the ability to distinguish an isoform from wild type, the greater the targeting ability to that isoform and the therapeutic benefit against disease that over expression or under expression of the isoform may cause. Further, the greater the ability to not interfere with wild type function, the less likely will be side effects and related complications of more general anti-coactivator therapy.

Please amend paragraph **[0088]**, as follows:

[0088] Thus, by designing siRNA molecules to target AIB1 or Δ3-AIB1, cellular levels of AIB1 can be reduced. The IC50 for this effect is 0.2 mM which indicates that it is possible to achieve tissue levels of siRNA for a therapeutic effect in vivo with only a few mg of siRNA per dose. The 5' region of the AIB1 mRNA was chosen as the initial target because it bears no

homology to other mRNA sequences as determined by a Blast search of the entire human genome. After a single liposome transfection into MCF-7 breast cancer cells this siRNA produced an up to 90% reduction in AIB1 protein levels as measured by Western blot analysis (Fig. 9). This effect was maintained for up to 72 hrs after addition of siRNA to the cells for a time period of 4 hours. Under this objective, alternative regions of AIB1 mRNA are targeted and tested to produce reductions in AIB1 gene expression at a lower IC50. To narrow down the chosen regions, areas common to Src-1 and Src-2 are excluded from this analysis as are common domain sequences such as the histone acetyl transferase domain and PAS/HLH sequence domains of known proteins. The remaining areas are used as candidates for a detailed Blast search. The best ten sequences are selected to make siRNA molecules. To specifically target [[?]] Δ3-AIB1, the junction of exons 2 to 4 is targeted which is a fusion that unique to this isoform (Fig. 9). A series of siRNA molecules between –13 and +13 relative to the junction (-9 /+13, -10 /+12, -11 /+11, -12 /+10, -13 /+9 etc.) are synthesized and their effect on target [[?]] Δ3-AIB1 relative to full-length AIB1 determined (Fig. 9b). Residual levels of both AIB1 isoforms are measured by Western blotting after electrophoretic separation of the isoforms on 4% polyacrylamide gels as described previously. Each siRNA molecule is tested at 5 concentrations of 1, 0.3, 0.1, 0.03 and 0.01 mM to determine the maximum reduction and the IC50. MCF-7 cells are the initial cell line used and confirmatory experiments are also run in T47D and MDA MB231 human breast cancer cells. These cells lines are chosen because they represent excellent model systems for human breast cancer: MCF-7 cells are responsive to estrogens, harbor the 20q AIB1 amplicon and express high levels of AIB1/[[?]] Δ3-AIB1 (14). T47D cells contain higher levels of AIB1 and [[?]] Δ3-AIB1 than normal mammary epithelium yet several fold lower levels than MCF-7 cells (14). They do not harbor the 20q amplicon, are estrogen responsive and, at least with respect to AIB1 expression levels, mimic a large portion of clinical breast cancers. MDA MB231 cells are chosen because they respond to growth factors for proliferation, are hormone-independent (ER-negative) and are a highly tumorigenic and metastatic in animal models. MDA MB231 cells are relatively invasive and have been well characterized in terms of both *in vitro* and *in vivo* invasive and chemotactic characteristics. This cell line responds to EGF and IGF with increased chemotaxis and to HGF with increased invasive capacity (49, 50). This cell line expresses intermediate levels of AIB1. Typically, the Prizm/Graphpad program is

used for curve fittings. For a 5-point siRNA dose response curve plus a negative control in triplicate, the cellular levels of AIB1 isoforms can easily be quantitated using serial Western blots as shown in Fig. 7. Once the concentration that achieves approximately 80% efficacy has been established for each siRNA molecule, the duration of the effect of each molecule can be examined at that concentration. Potency and thus, the drug concentration needed for the targeted effect level, can be tested empirically and those siRNAs chosen for use in animals that show the best dose/time/response (lowest dose to get a 3 day effect >50%). In the experiment described in Fig. 7, it was found that the effects of the siRNA directed at AIB1 were preserved for at least 72 hours. Some reports have claimed that siRNA effects can be prolonged to >100 hours in vitro after a single administration (51). Therefore, time course experiments are run for up to 120 hours with the optimal dose of each siRNA to determine the duration of the RNA silencing and whether this differs amongst the siRNAs chosen.

Please amend paragraph **[0089]**, as follows:

[0089] To determine if siRNA-induced reduction of cellular levels of AIB1 or [[?]] Δ3-AIB1 can change the phenotype of breast cancer, cell lines are tested to examine if siRNA-mediated depletion of AIB1 or [[?]] Δ3-AIB1 result in estrogen or growth factor-induced changes in cellular proliferation and soft agar colony formation. It is first determined if there are changes in doubling time or in colony formation under anchorage-independent growth conditions in soft agar. In addition to spontaneous growth, cells are cultured in serum only or EGF (10 to 100 ng/ml), IGF-1 (10 to 100 ng/ml), heregulin (10 to 100 ng/ml), HGF (hepatocyte growth factor; 10 to 100 ng/ml). The growth responses after 2, 4, 6 and 8 days are compared in the presence or absence of siRNA (+/- growth factor) at the optimal concentration of siRNA previously determined. For the growth assays, it is only necessary to test the siRNA molecules that have had effective and long lasting effects as well as those that are specific for [[?]] Δ3-AIB1. A single addition of siRNA is used initially and several dosings of siRNA (every other day) for those conditions that showed effects only after the initial dosing. For the soft agar assays, cells are cultured in the presence or absence of EGF, IGF-1, heregulin, HGF or serum (concentrations as above) with or without siRNA (only one dose is used initially). After at least 7 days of incubation, colonies are counted with an image

analyzer. In addition, experiments with the MCF-7 and T47D cells can include estrogen to determine whether AIB1 contributes to synergism between growth factor and estrogen-mediated signaling.

Please amend paragraph **[0093]**, as follows:

[0093] The deletion of the AIB1 (CIP) gene in mice resulted in a surprising phenotype where mice had reduced overall growth due, in part, to reduced serum IGF levels (53, 54), but mainly because IGF signaling was reduced in $AIB1^{-/-}$ cells (53). Serum induction of proliferation in $AIB1^{-/-}$ cells was unaltered (53). In human breast cancer cells it was found that the isoform [[?]] Δ3-AIB1 can strongly potentiate EGF signaling (48) and similar to the AIB1 knock-out in mice. The reduction of overall AIB1 levels in MCF-7 cells inhibits IGF-1 and heregulin induced growth, but does not affect growth under control conditions, i.e. in the presence of serum (Fig. 8). Thus, for the targeting of AIB1, siRNA against isoforms of AIB1 is a viable therapy for the treatment of human breast cancer.

Please amend paragraph **[0094]**, as follows:

[0094] These observations indicate that a central role of AIB1 in a defined number of signaling pathways induced by growth factors that are known to play pivotal roles in the malignant progression of breast cancer. Over expression of growth factor receptors (HER-2/neu and EGFR) are correlated with a more aggressive phenotype and with a decreased responsiveness to antiestrogen therapy. Thus, over expressed AIB1 and, in particular, the isoform [[?]] Δ3-AIB1 is a master regulator that drives a more aggressive phenotype of breast cancer and AIB1 and [[?]] Δ3-AIB1 can be used as therapeutic targets in breast cancer. The validity of AIB1 as a therapeutic target is based on the facts that: (i) AIB1 is a rate-limiting regulator for estrogen and growth factor signaling in breast cancer; (ii) AIB1 is over expressed selectively in breast cancer cells and not in non-transformed tissues; (iii) the knock-out of the AIB1 gene in mice indicates that the side-effects of AIB1 reduction are limited to the growth phase of the body before adulthood and to the reproductive system indicating that that in an adult, target-specific side effects are very likely small;

(iv) selective reduction of the [[?]] Δ3-AIB1 isoform is possible by generating siRNAs that target the splice junction of exons 2/4 (see Fig. 9b) and, thus, only deplete this isoform. Accordingly, targeting [[?]] Δ3-AIB1 isoform is more effective against cancer than targeting full-length AIB1. As shown in Fig. 1a, the [[?]] Δ3-AIB1 isoform is selectively over expressed in breast cancer cells and cancer tissues from patients and is barely detectable in normal breast tissues. In addition, this isoform is significantly more potent than full-length AIB1 on a molar basis and thus contributes to a large percentage of AIB1 effects on growth and proliferation in cancerous tissue; and (v) because AIB1 is selectively over expressed in tumor tissues, the side effects from therapeutic targeting of the [[?]] Δ3-AIB1 isoform are expected to be minimal.

Please amend paragraph **[0097]**, as follows:

[0097] Utilizing the most potent of the AIB1 and [[?]] Δ3-AIB1 siRNAs identified, one can determine a regimen of administration of siRNA to animals to produce a sustained reduction of AIB1 in animal tumors and ultimately an anti-tumorigenic effect *in vivo*. Distribution and approximate tissue concentration is determined after siRNA administration. From this, an optimal dose and dosing interval is assessed for the animal. Fluorescently tagged (FITC) active siRNA molecules are prepared and injected ip or iv into nude mice that carry at least one MCF-7 xenograft tumor each. The tissue levels of siRNA are estimated in the tumor, liver, muscle, kidney and brain at several time points after injection. From cell culture studies, it was shown that tissue concentrations of 0.1 to 0.3 micromolar can reduce AIB1 protein significantly (see Fig. 7). To achieve this concentration *in vivo,* one would need to inject approximately 2 to 6 mg of siRNA i.p. per mouse. To maintain these levels will depend on the half life of the siRNA liposome complex *in vivo* and this is determined using fluorescein conjugated molecules. Animals are sacrificed at 6, 12, 24, 48 and 72 hours of i.p. injection of the tagged siRNA and the levels of fluorescein conjugated molecule determined by fluorescence detection in the homogenates of liver, kidney, muscle, brain and tumor. FITC labeled siRNA is imaged after treatment of MCF-7 cells *in vitro*. In parallel with the measurement of siRNA levels by fluorescence, AIB1 levels are measured in the tumors by Western blot. Thus, the time course of drug concentrations is compared with the time-effect relationship. After analysis of the first series with a single intraperitoneal dose, the dose interval

(bid, tid etc.) and/or dose level or even the route of administration (intratumoral, i.v.) can be modulated if that promised better efficacy based on AIB1 levels. To study efficacy of siRNA not only on the target gene, but on tumor growth, MBA-MB-231, MCF-7 or T47D cells are implanted into mammary glands of athymic nude mice and their growth followed. In the first series of experiments, systemic administration of siRNA, most likely twice per week i.p., initiates after the formation of palpable tumors. Five mice/group and two tumor inoculum sites per mouse comprise one treatment or one control arm. One can start with two to three i.p. doses of siRNA per week, but this would be modified to more frequent (daily) or less frequent (weekly) dosing as time course studies require. Dosing is continued for the whole duration of the experiment. Tumor growth is monitored for up to two months following implantation and tumor size estimated from the product of perpendicular diameters of the tumor (twice weekly). Tumors are stained for proliferation (by PCNA staining) and mitotic cells, apoptosis (TUNEL staining) and the number of blood vessels. Statistical considerations and evaluations are well known. Targeting AIB1 by regulatable ribozymes in MCF-7 xenograft tumors will not only delay tumor growth, but will induce complete regression of implanted tumors due to siRNA. Treatment with siRNA would be stopped after tumor regression and patients is followed to monitor for possible tumor re-growth. The effect of siRNA is tested on large established tumors of approximately 1 cm in their largest diameter. The extent to which apoptosis may be induced can be assayed by harvesting and staining of the tumor tissues after 2 to 3 weeks of siRNA treatment.